A model for repair of radiation-induced DNA double-strand breaks in the extreme radiophile *Deinococcus radiodurans*

Kenneth W. Minton and Michael J. Daly

Summary

The bacterium *Deinococcus* (formerly *Micrococcus*) *radiodurans* and other members of the eubacterial family Deinococcaceae are extremely resistant to ionizing radiation and many other agents that damage DNA. Stationary phase *D. radiodurans* exposed to 1.0-1.5 Mrad γ-irradiation sustains >120 DNA double-strand breaks (dsbs) per chromosome; these dsbs are mended over a period of hours with 100% survival and virtually no mutagenesis. This contrasts with nearly all other organisms in which just a few ionizing radiation induced-dsbs per chromosome are lethal. In this article we present an hypothesis that resistance of *D. radiodurans* to ionizing radiation and its ability to mend radiation-induced dsbs are due to a special form of redundancy wherein chromosomes exist in pairs, linked to each other by thousands of four-stranded (Holliday) junctions. Thus, a dsb is not a lethal event because the identical undamaged duplex is nearby, providing an accurate repair template. As addressed in this article, much of what is known about *D. radiodurans* suggests that it is particularly suited for this proposed novel form of DNA repair.

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What is *Deinococcus radiodurans?*

This remarkable bacterium was originally isolated in Oregon by Anderson and coworkers in 1956 from radiation-sterilized canned meat that had undergone spoilage[1]. Culture yielded a red-pigmented, nonsporulating, nonpathogenic, Gram$^+$ bacterium that was extremely resistant to the lethal and mutagenic effects of ionizing radiation and to many other agents that damage DNA[2-4]. Subsequently, four additional deinobacterial species were isolated from diverse sources ranging from irradiated Bombay duck[5] to weathered granite in Antarctica[6]. These closely related species, all of which are extremely DNA-damage-resistant, have been grouped with *D. radiodurans* to form one of the ten known eubacterial families, Deinococcaceae[4]. Because there has been no systematic search for the deinococci, their natural habitat, or niche, has not been identified. With respect to evolution, as determined by 16S rRNA sequences, the deinobacteria are extremely distant from any well-characterized species, being just as far from *Escherichia coli* as from *Bacillus subtilis*[7]. To date, the deinobacterial species are the most ionizing- and UV-radiation resistant organisms known[3] (Fig. 1).

Radiation-induced dsbs are hazardous to your health, unless you are *D. radiodurans*

Ionizing radiation-induced dsbs pose a formidable challenge for cellular DNA repair processes because both strands of the double helix are broken[8]. Repair of these lesions is more difficult than damage that affects only one strand [e.g. ionizing radiation-induced DNA single-strand breaks (ssbs) or damage to DNA bases][8], which can be repaired by local excision of the damaged single strand while the complementary undamaged strand provides a template to guide accurate resynthesis at the repair site[9]. In contrast, DNA damage-induced dsbs provide little in the

is a variety of MNNG-mutagenized strains that are ionizing radiation-sensitive[3,23]. These strains are proving useful in isolating genes by complementation assays that restore wild-type ionizing radiation-resistance[23-25]. To date, two genes have been identified and characterized in terms of both their DNA sequence and corresponding proteins; the remaining mutant strains are subject to ongoing research. These two genes are the deinococcal *polA*$^+$ (DNA Pol I) and *recA*$^+$ (RecA protein) genes[24,25]. Deinococcal strains that are *polA*$^-$ are very sensitive to a variety of DNA damaging agents including ionizing radiation[24]. However, when the *E. coli polA*$^+$ gene was expressed in *polA*$^-$ *D. radiodurans* there was complete restoration of wild-type deinococcal resistance to all tested forms of damage, including ionizing radiation[26]. This observation indicates that the deinococcal DNA Pol I is not uniquely qualified, since *E. coli* DNA Pol I can do the same job, i.e. DNA Pol I is necessary, but not sufficient[26].

The evidence with respect to the deinococcal RecA homologue is quite different from the DNA Pol I homologue. Strains defective in the *recA* gene are the most ionizing radiation-sensitive deinococcal strains discovered to date, approaching the radiation sensitivity of *E. coli recA*$^+$ cells (Fig. 1). Expression of the *Shigella flexneri recA* protein in *D. radiodurans recA*$^-$ cells results in no increase of DNA damage resistence*, even when expressed at high levels as determined by western blotting (K. W. Minton and M. J. Daly, unpublished results). The reciprocal experiment, i.e. expression of the *D. radiodurans recA* gene in *recA*$^-$ *E. coli*, results in severe toxicity or death of the *E. coli* recipient, even at low levels of expression[25]. This is unusual, since the majority of *recA* genes of various bacterial species typically complement *E. coli recA* strains, and *vice versa*[13].

The central hypothesis

It is proposed here that in *D. radiodurans* pairs of double-helical chromosomes are closely associated with each other. The presence of 4-10 chromosomal copies is not in itself nearly sufficient to impart to *D. radiodurans* its DNA damage-resistance[28]. For example, all eukaryotic cells in G_2 are tetraploid, but very damage-sensitive. Bacteria with many chromosomes, such as *M. luteus* and *M. sodonensis*, are also very sensitive[3]. *Azotobacter vinelandii*, that contains 40 to 80 chromosomes per cell[29,30], is UV-sensitive[31]. These observations address the question of why diploid or polyploid organisms other than the deinobacteria are *not* resistant to radiation. We suggest that *D. radiodurans* makes use of redundant information in a manner that these other organisms do not.

While investigating recombinational repair in *D. radiodurans-E. coli* shuttle plasmids damaged *in vivo* by ionizing radiation[17,18], observations were made in both wild-type and *recA D. radiodurans*, compatible with the presence of four-stranded junctions (Holliday junctions) between plasmids (K. W. Minton and M. J. Daly, unpublished results). We have so far no evidence for such junctions when these same plasmids were purified from *E. coli* of either genotype. This possibility has led us to speculate that, like plasmids, the chromosomes of *D. radiodurans* might also be linked by Holliday junctions, giving rise to the following hypothesis:

Specifically, that pairs of the deinococcal chromosomes are joined to each other at thousands of sites by four-stranded junctions (Holliday junctions).

A refresher course in four-stranded junctions

Four-stranded junctions, also known as Holliday junctions[32,33], are generally thought of as intermediate structures in genetic recombination occurring between homologous or identical regions of two DNA duplexes (Fig. 2). Their presence in both prokaryotes and eukaryotes[34,35] and their properties *in vitro*[36] have been extensively studied, and they are an integral part of virtually all models of genetic recombination. *In vitro* studies on Holliday junctions indicate that they possess no single-stranded character and all bases are paired[36] (as in Fig. 5, top). They are able to move freely in regions of identity since there is no net gain or loss of base pairs, and their movement is typical of a 'random walk'[34,37,38]. Migration of Holliday junctions, either spontaneously or accelerated enzymatically, is referred to as 'branch migration' (Fig. 2). Movement in a given direction is greatly impeded if branch migration encounters heterologies including base mismatches, unless the junction is enzymatically driven across such heterologous regions at the expense of either ATP or dATP[37,38].

Why are pre-existing Holliday junctions useful in repair of dsbs?

With respect to the ability of *D. radiodurans* to repair myriad dsbs, the essential property endowed by the presence of numerous persistent Holliday junctions is that their occurrence between homologous regions serves to organize the genetic material in space such that pairs of chromosomes of *D. radiodurans* are aligned. If so, then the 'search for homology'[13] for repair of dsbs becomes simpler due to a pre-existing alignment. The alignment of homologous chromosomal regions is often tacitly

*The *S. flexneri* RecA protein is identical to the *E. coli* RecA protein[13,27]. The *S. flexneri recA* gene was used for gene expression in *D. radiodurans* because of convenient restriction sites (and was a gift of K. McEntee).

configuration, virtually all Holliday junctions would be antiparallel[36]. However, the relatively small difference in free energy could be overcome readily by a variety of factors, including restraining DNA binding proteins or prohibitive transition energies. In the current context, 'antiparallel' means that the two *duplexes* are oriented in opposite directions with respect to each other, and 'parallel' indicates alignment of two duplexes in the same direction[36,38,41]. Both branch migration and the proposed model of alignment of chromosomes by Holliday junctions are much easier to reconcile as occurring between helices that are both parallel. However, the antiparallel configuration might also be acceptable, since this would call only for small reversals localized to the sites of the Holliday junctions themselves.

While the effect of Mg^{2+} on four-stranded junctions has been studied extensively, the consequence of Mn^{2+} has not. This overlooked cation is of special significance to *D. radiodurans* because this organism appears to be unique in that it has an exceptionally high intracellular Mn^{2+} content, 100-fold greater than in *E. coli*[42]. Furthermore, Mn^{2+} binds DNA with an affinity about 5-fold greater than Mg^{2+}[43]. Localization of this Mn^{2+} has determined that DNA is the prime repository, and it is possible to estimate that there is approximately one Mn^{2+} ion bound by DNA per every 7 base pairs. The effect of this large amount of associated Mn^{2+} on the structure of chromosomes and Holliday junctions, while unknown, may be important in several ways, including the exact configuration of the stacked X, the free energy and transition energy of parallel and antiparallel configuration, and the distance of approach of the paired double helices, due to the enhanced backbone shielding.

Semiconservative DNA replication and preservation of Holliday junctions

How are thousands of Holliday junctions preserved during semiconservative DNA replication? Are they first resolved, followed by chromosomal replication, and then finally reintroduced by RecA and associated proteins? This is an issue to our knowledge that has never been addressed[44], and the mechanism suggested above seems ponderous indeed. Instead, we suggest that *D. radiodurans* is capable of copying Holliday junctions along with the rest of the chromosome during semiconservative DNA synthesis. The ability to do so is not inherent in what is already known regarding replicative DNA synthesis pathways[44], but can be deduced, as follows.

With simultaneous initiation of semiconservative DNA synthesis at each of the chromosomal origins of replication in a given linked pair of chromosomes (Fig. 4), the replication forks will encounter a given Holliday junction simultaneously (assuming the junction is not resolved or pushed

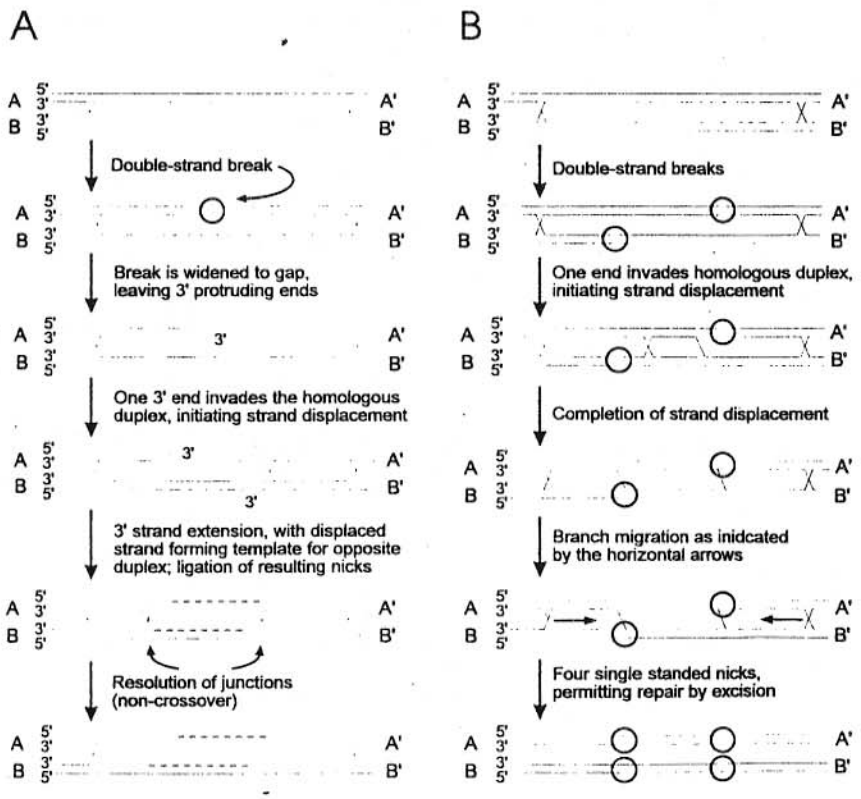

Fig. 3. (A) A model for dsb repair adapted from proposed models of dsb-induced generalized homologous recombination. (B) A model for dsb repair adapted from models of ssb-initiated homologous recombination.

cation in the absence of RecA does not appear to require a complicated solution.

Nevertheless, Holliday junctions will be lost over time due to resolvase(s), DNA repair or annihilation when two like Holliday junctions meet. It may be the case that RecA amounts that are less than detectable, are sufficient to mediate initiation of new Holliday junctions. A novel possibility is that *D. radiodurans* harbors an enzyme of the λ integrase family, which produces Holliday junctions at specific sites as its primary activity[45]. *D. radiodurans* contains numerous repeated sequences of 100-200 bp of unknown function[46], and these might serve as integrase-specific sites.

Predictions and experimental approach

This model makes two straightforward predictions: First, electron microscopic examination of adequately spread chromosomal DNA from *D. radiodurans* should reveal evidence of Holliday junctions or possibly other structures linking chromosomes or chromosomal fragments; and second, that chromosomal restriction fragments linked by Holliday junctions should migrate aberrantly and, therefore, be detectable by neutral agarose gel electrophoresis and Southern blotting.

Holliday junctions could be lost during purification of chromosomal DNA processes related to branch migration, e.g. by migration off the ends of the two linked double-helices. One remedy for this potential complication could be to treat the cells *in vivo* with the DNA crosslinking agent trimethylpsoralen plus UV_{360}, immediately before isolation of the DNA. This technique is commonly used to generate interstrand crosslinking *in vivo*[47]. Crosslinking should inhibit branch migration of Holliday structures because a junction cannot proceed in a given direction if one of the two double-helices contains an interstrand crosslink at that site.

Results of studies such as those described above will be presented in due course.

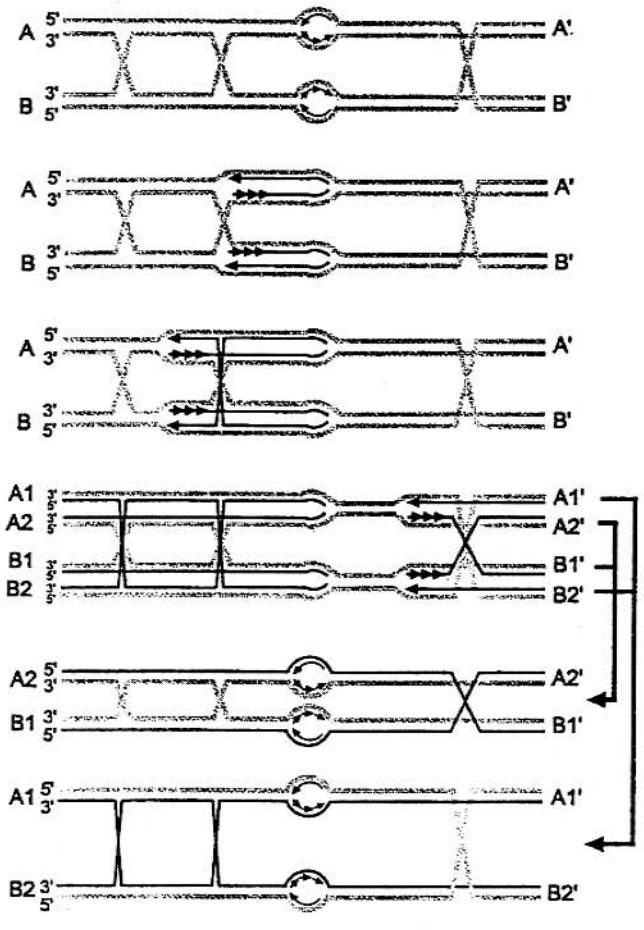

Fig. 6. A cartoon of two complete double-helical chromosomes attached to each other by three Holliday junctions. The parental chromosomes are shown in broad grey lines, while the newly synthesized DNA is represented by thin black lines. Arrowheads as in Fig. 4.

Acknowledgements

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